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Quantitative Analysis of Cytomegalovirus Load Using a Real-Time PCR Assay

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A novel real-time PCR assay system was developed to quantify the cytomegalovirus (CMV) genome load. The real-time PCR assay could detect from 6 to over 10^6 copies of CMV-DNA with a wide linear range. The virus load of immunocompromised patients with symptomatic CMV infections was quantified and compared to that of asymptomatic ones. In symptomatic patients, all 17 peripheral blood leukocytes were positive for CMV DNA, and its mean value was $10^{3.3}$ copies/10^6 cells. On the other hand, only 9 of 38 samples (24%) were positive in the asymptomatic patients, and its mean titer was lower ($10^{2.0}$ copies/10^6 cells) than that of the symptomatic group ($P = 0.002$). In plasma, the virus genome was detected in 13 out of 17 samples from symptomatic patients (76%), and its mean value was $10^{4.0}$ copies/ml. In contrast, for the asymptomatic group, only one out of 36 samples were positive (3%). Finally, this system was used to monitor two patients with CMV infections serially. The CMV DNA copy number changed with their clinical symptoms and anti-CMV therapy, and virtually paralleled the result of the pp65 antigenemia assay in both cases. In one patient with the cord blood transplantation, however, the CMV DNA became positive faster than the antigenemia assay. These results indicate that this assay is sensitive and useful for estimating the CMV genome load not only in peripheral blood leukocytes but also in plasma. It can be very helpful for diagnosing CMV-related diseases and monitoring the virus load in patients with CMV infections. ***J. Med. Virol. 60:455–462, 2000.***

KEY WORDS: CMV; TaqMan; quantitative PCR; pp65-antigenemia assay

INTRODUCTION

Cytomegalovirus (CMV) is an important pathogen in immuno-compromised patients, such as patients with AIDS or recipients of bone marrow or organ transplants. This virus is the most common cause of intrauterine infections, and sometimes causes a life-threatening cytomegalic inclusion disease. Surviving neonates develop subsequently significant neurological sequelae or deafness [Stagno, 1995]. The early and accurate diagnosis and monitoring of CMV infection status is one of the principal ways to manage these patients [van der Meer et al., 1996; Numazaki and Chiba, 1997; Hebart et al., 1998; Whitley et al., 1998]. Because the primary CMV infection usually results in a latent life-long infections, however, and even healthy individuals secrete CMV in their saliva or urine [Mocarski, 1996], the detection of CMV does not always indicate a CMV-related diseases.

PCR is now used to diagnose and monitor various viral infections; however, the traditional, qualitative PCR assay is too sensitive to monitor and diagnose CMV-related diseases, and has some difficulty in deciding when to start and terminate anti-viral therapy [Gerna et al., 1991]. Although quantitative analysis is important to diagnose or monitor virus-related disease [Shibata et al., 1992; Ando et al., 1993; Shinkai and Spector, 1995; Yamamoto et al., 1995; Boeckh and Boivin, 1998], methods such as competitive-quantitative PCR assay or quantitative viral isolations take a long time and are labor intensive. They are not convenient for rapid-decision making in clinical situations. The antigenemia assay, one of the methods used

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to quantify CMV, is now recognized widely as useful for diagnosing and monitoring CMV diseases in clinical situations [Grossi et al., 1995; van der Meer et al., 1996]. This method is limited, however, because only viruses associated with cells are detectable, and viruses free in the plasma, urine, or cerebrospinal fluid can evade this assay. Furthermore, this assay cannot be carried out when patient's leukocyte count is low [Boeckh et al., 1997].

Recently, a novel "real-time" quantitative PCR method was developed [Heid et al., 1996]. This method measures the accumulation of PCR products with a fluorogenic probe and real-time laser scanning in a 96-well plate, and has a very wide dynamic range [Kimura et al., 1999]. Because this assay does not require additional handling, such as electrophoresis, after the sample is drawn, much faster assays with a higher throughput are possible. This assay has been applied to estimate the viral load for diagnosing or monitoring several viral infections [Morris et al., 1996; Kennedy et al., 1998; Kimura et al., 1998; Kimura et al., 1999]. In this study, a quantitative system was developed for estimating CMV load using this technique. Using this assay, the CMV viral load in patients with CMV infection was quantified and compared the viral load between patients with CMV-related diseases and asymptomatic infections. Finally, this assay system was applied for monitoring clinical cases of CMV infection in comparison with the antigenemia assay.

MATERIALS AND METHODS

Study Population

Fifty patients, who were seropositive for CMV, were enrolled in this study. They were categorized into 4 groups as follows.

Immunocompromised-Symptomatic Group. This group consisted of five patients (3 boys and 2 girls). They were from 1 month to 9 years old (median, 8 years) and their illnesses included 1 case of severe combined immunodeficiency with intractable CMV-retinitis, 1 renal transplant recipient with CMV-retinitis, 1 liver transplant recipient with interstitial pneumonitis and liver dysfunction, and 2 cord-blood transplant recipients who showed CMV-enteritis.

Immunocompromised-Asymptomatic Group. Twelve patients were included in this group (5 males and 7 females). They were from 1 month to 39 years old (median, 10.5 years). Seven patients were hematopoietic stem cell transplant recipients and 5 were liver transplant recipient. These patients were examined because they were seropositive for CMV before transplantation and they were at high risk for CMV reactivations. None of them showed CMV-related symptoms after transplantation.

Immunocompetent-Symptomatic Group. Thirteen patients were included in this group (8 boys and 5 girls). They were 1 day to 1 years old (median, 3 months). They were 5 patients with congenital infections, 7 with infantile CMV hepatitis, and one with infectious mononucleosis-like syndrome.

Immunocompetent-Asymptomatic Group. This group consisted of 20 patients (14 boys and 6 girls). They were 2 months to 16 years old (median, 4.5 months). Although either a primary infection or reactivation was suspected serologically or virologically, CMV-related symptoms were not observed in these patients. By definition, primary CMV infection occurred when patients had seroconversion to CMV or had anti-CMV IgM antibody, whereas reactivation of CMV occurred when a significant rise in anti-CMV antibody was observed or CMV was detected in blood or urine of patients.

EDTA-treated whole blood and urine were collected from these patients and used for the real-time PCR assay. The blood samples were also used for the pp65 antigenemia assay.

Sample Preparation

EDTA-treated peripheral blood was collected from patients and controls after informed consent was obtained. Peripheral blood leukocytes (PBLs) were prepared in the following way. Two ml of peripheral blood were treated with erythrolysis solution (0.83% NH_4Cl, 0.1% $KHCO_3$, 0.0037% EDTA-Na_2) to lyse the erythrocytes, and incubated on ice for 20 minutes. Then, the leukocytes were collected by centrifugation at 400 × *g*, washed with phosphate buffer saline (PBS), and resuspended in PBS. Mononuclear cells (MNCs) and polymorphonuclear cells (PMNCs) were prepared from the suspension of PBLs by Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) density centrifugation. Although a small number of contaminating cells was observed microscopically, the objective cells dominated each fraction.

For the PCR assay, DNA was extracted from 10^6 PBLs, MNCs, or PMNCs using a QIAamp Blood Kit (QIAGEN GmbH, Hilden, Germany), eluted in 50 μl of distilled water and stored at −30°C until analysis. Plasma was separated from EDTA-treated whole blood by centrifugation. Heparinized blood was not used because heparin inhibits PCR [Kimura et al., 1999]. DNA was extracted from 200 μl of plasma in the same manner. DNA was also extracted from 140 μl of urine with a QIAamp Viral RNA kit (QIAGEN Inc) according to the manufacturer's instructions. Totally, 84 samples of PBLs, 79 samples of plasma, and 21 samples of urine were analyzed.

Real-Time Quantitative PCR Assay

The PCR primers are from the immediate early (IE) gene [Akrigg et al., 1985]. The upstream primer was 5′-GACTAGTGTGATGCTGGCCAAG-3′ and the downstream primer was 5′-GCTACAATAGCCTCTTCCTCATCTG-3′. A fluorogenic probe (5′-carboxyfluorescein–AGCCTGAGGTTATCAGTGTAATGAAGCGCC-3′) was located between the PCR primers [Heid et al., 1996]. The PCR reaction was carried out using a TaqMan PCR kit (PE Applied Biosystems, Foster City, CA), as described previously [Heid et al., 1996; Kimura et al., 1999]. Briefly, 10 μl of DNA extraction

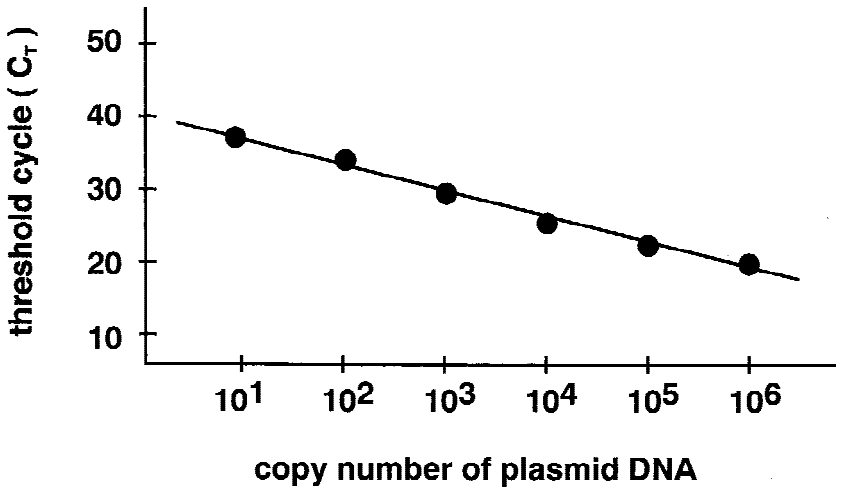

Fig. 1. Standard curve for real-time PCR. Serially diluted pGEM-IE plasmid was amplified and analyzed in real-time using a model 7700 Sequence Detector. The threshold cycle (C_T) values were plotted against copy number to construct the standard curve.

solution from the samples were added to a PCR mixture containing 10 mM Tris (pH 8.3), 50 mM KCl, 10 mM EDTA, 5 mM $MgCl_2$, 100 μM dATP, dCTP, dGTP, dTTP, 0.2 μM each primer, 0.1 μM fluorogenic probe, and 1.25 U of AmpliTaq Gold (PE Applied Biosystems). After activation of the AmpliTaq Gold for 10 min at 95°C, 50 cycles of 15 sec at 95°C and 1 min at 62°C were carried out in a Model 7700 Sequence Detector (PE Applied Biosystems). Real-time fluorescent measurements were taken and a threshold cycle (C_T) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 × SD of the base line). For a positive control, a plasmid that contained the IE gene was constructed from pGEM-T vector (Promega, Madison, WI), and called pGEM-IE. A standard graph was constructed using the CT values obtained from serially diluted pGEM-IE. The C_T values from the clinical samples were plotted on the standard curve, and the copy number was calculated automatically using Sequence Detector v1.6 (PE Applied Biosystems), a software package for data analysis. Samples were defined as negative when the C_T value exceeded 50 cycles. The DNA copy numbers of PBLs, PMNCs, and MNCs are expressed per 10^6 cells. The results for the plasma and urine are expressed per ml.

pp 65-CMV Antigenemia Assay

A pp65 antigenemia assay was carried out using a kit (Mitsubishi Chemical, Tokyo, Japan), according to the manufacturer's instruction. Briefly, 1.5×10^5 PBLs, that include both neutrophils and MNCs, were cytospun to a glass slide and fixed. Primary pp65 monoclonal antibodies, C10 and C11, were added on the slides and incubated for an hour [Veal et al., 1996; Lazzarotto et al., 1998]. After washing, secondary antibodies, alkaline phosphatase labeled anti-mouse and anti-goat polyclonal antibody solution, were added, and incubated for 1 hr. After washing, the substrates were added and developed for 15 min. Cells stained red or violet after counter-staining with hematoxylin were defined as positive for pp65 antigen, and all the positive cells on each glass slide were counted. The number was expressed per 5×10^4 PBLs.

Statistical Analysis

The software package StatView J 4.02 (Abacus Concepts Inc., Berkeley, CA) was used for data analysis. The regression analysis was used to compare the real-time PCR and antigenemia assays. Student's *t*-test was used to compare the mean CMV DNA copy number in each group. Fisher's exact test was used to compare the CMV DNA detection rate in each group.

RESULTS

Establishing a Real-Time PCR Assay for Quantifying CMV-DNA

pGEM-IE diluted serially was tested using the real-time PCR assay, and a standard curve was constructed from the C_T values obtained using these positive controls. The control plasmid produced a wide linear range from 10 copies to 1×10^6 copies (Fig. 1). A minimum of 6 copies of the plasmid could be detected by the system (data not shown).

To confirm the specificity of the primers and probe, other human herpesviruses (herpes simplex virus type 1 and 2, varicella-zoster virus, human herpes virus 8, and Epstein-Barr virus), and PBLs, plasma, and urine from CMV-seronegative individuals were tested by this system. All the results were negative for CMV DNA.

It was estimated whether the MNCs or PMNCs fraction had a larger amount of virus DNA, to determine which fraction should be used for further experiments. The peripheral blood from 2 patients with severe symptomatic CMV infections was analyzed. In one patient, the CMV DNA copy numbers in MNCs and PMNCs were $10^{4.4}$ and $10^{4.9}$ copies/10^6 cells, respectively. In the other patient, they were $10^{2.2}$ and $10^{1.8}$ copies/10^6 cells, respectively. The virus genomes existed in both fractions. At the same time, each fraction was analyzed using the pp65 antigenemia assay, and pp65 antigen was detected equally in each fraction. Because both the MNCs and PMNCs fractions contained CMV DNA and

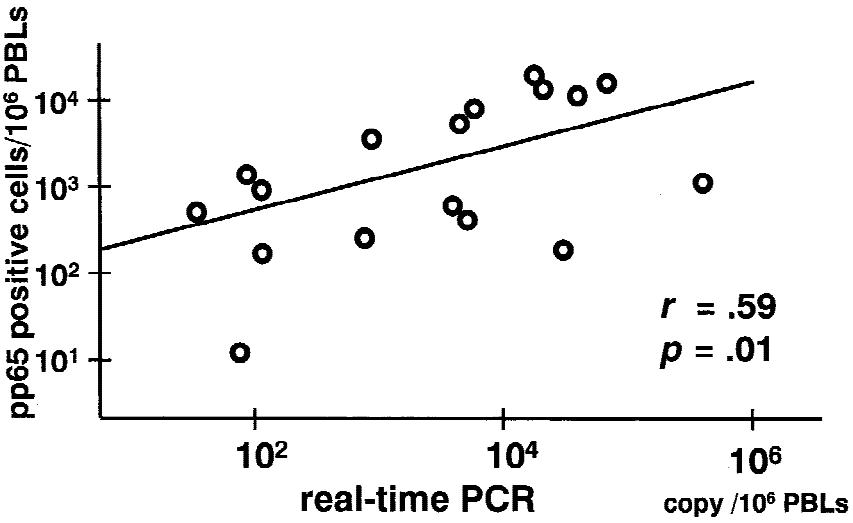

Fig. 2. Correlation between copy number of CMV-DNA and pp65-positive cell count in peripheral blood leukocytes (PBLs). PBLs were obtained from patients with symptomatic CMV disease and 17 samples were analyzed using both the real-time PCR and antigenemia assay. The copy numbers of CMV-DNA measured by the real-time PCR assay and pp65-positive cell numbers by the antigenemia assay were plotted and the correlation coefficiency (r) was calculated.

the pp65 antigen, PBLs that contained both fractions were used for further analysis. Furthermore, preparing PBLs was easier and has less handlings than PMNCs or MNCs. It was considered that such method with less handlings could reduce the risk of PCR contamination.

Comparison of the Virus DNA Load and the pp65 Antigenemia Assay

The correlation of the real-time PCR assay and the pp65 antigenemia were investigated in blood samples from patients with symptomatic CMV diseases. Seventeen PBLs samples were obtained and analyzed using both the real-time PCR and antigenemia assays (Fig. 2). The correlation between the CMV DNA copy number and the pp65-positive cell count was statistically significant (correlation coefficient = 0.59, $P = 0.01$).

Quantification of CMV-DNA in Patients With Symptomatic CMV Infection and Comparison with Asymptomatic Patients

Next, the virus load of samples from patients was compared with symptomatic CMV infections and that of asymptomatic ones in either immunocompromised or immunocompetent group by means of the real-time PCR assay.

For the samples of PBLs, all 17 samples (100%) were positive for CMV DNA in the immunocompromised-symptomatic group, and the mean copy number of CMV DNA was $10^{3.3}$ copies/10^6 cells (Fig. 3A). On the other hand, in the asymptomatic group, the virus DNA was detected in 9 out of 38 samples (24%), and the mean copy number was smaller than that in the symptomatic group ($10^{2.0}$ copies/10^6 cells, $P = 0.002$). In the immunocompetent-symptomatic group, CMV DNA was detected in 8 out of 11 samples (73%), and its mean copy number was $10^{2.1}$/10^6 cells. In the asymptomatic group, however, no samples out of 18 were positive for CMV DNA with this assay (Fig. 3A).

In plasma, 13 out of 17 samples in the immunocompromised-symptomatic group were positive for the virus genome (76%), and the mean copy number was $10^{4.0}$/ml. On the other hand, only one out of the 36 samples from asymptomatic group was positive (3% in Fig. 3B). In the immunocompetent-symptomatic group, only one out of 7 samples was positive for the CMV DNA in plasma (14%). None out of 19 samples from asymptomatic group were positive for the virus genome (Fig. 3B).

The urine samples of the immunocompetent group were examined (Fig. 3C). Six out of 9 samples from the symptomatic group and 9 out of 12 samples from asymptomatic one were positive for the virus genome. Although the rate of detection was similar in the two groups (67% vs. 75%, $P = 0.7$), the mean of CMV DNA copy number in positive samples was significantly higher ($10^{5.7}$ vs. $10^{3.8}$/ml of urine,$P = 0.003$).

Serial Monitoring of CMV-Load With the Real-Time PCR Assay

Patients who had either intractable CMV diseases or high risks for CMV infections using the real-time PCR assay were examined. The virus load in the patients' PBLs and plasma was monitored and compared the results with the pp65 antigenemia assay in PBLs. Two representative cases are shown as follows.

Patient A was a 4-month-old boy with severe combined immuno-deficiency. He had intractable CMV-retinitis and hepatitis from the time of first visit to the hospital and was treated with ganciclovir and foscarnet (Fig. 4A). Despite the antiviral therapy, the CMV-retinitis progressed fulminantly. Allogenic bone marrow transplantation was carried out to correct his primary disease, but he died of severe veno-occlusion disease on Day 15 after transplantation. The CMV DNA copy number in the PBLs and plasma and pp65 positive cells all roughly paralleled his clinical course, although these parameters deviated slightly after initiating the conditioning therapy for his bone marrow transplantation. The number of pp65 positive cells decreased more than the CMV genome load in the PBLs or plasma.

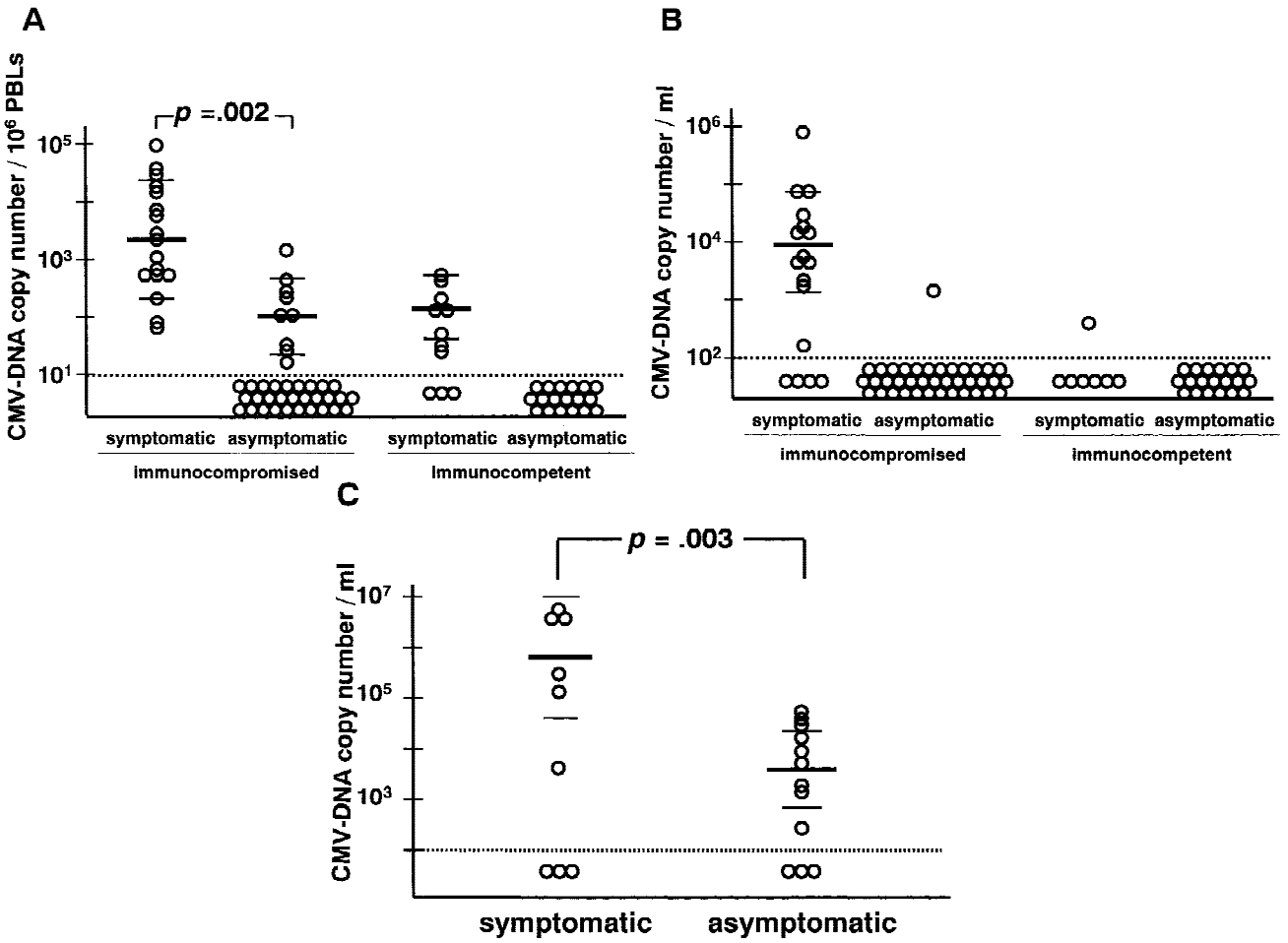

Fig. 3. Comparison of virus load between symptomatic and asymptomatic CMV infections. DNA was extracted from peripheral blood leukocytes (PBLs), plasma, and urine from immunocompromised or immunocompetent patients. Copy number of CMV DNA was determined by the real-time PCR assay. Results were compared between patients with symptomatic CMV infections and asymptomatic patients without CMV-related disease. Each copy number was shown per 10^6 PBLs, or per ml of plasma or urine, respectively. Bars show the mean and standard deviation for each group. Dotted lines of each panel show the threshold of each sample. (A) Comparison of CMV DNA copy number in PBLs. (B) Comparison of CMV DNA copy number in plasma. (C) Comparison of CMV DNA copy number in urine. Urine was only available for the immunocompetent patients.

Patient B, was a 14-year-old girl with acute myelogenic leukemia, who had a cord-blood transplantation. Before transplantation, a very small amount of CMV DNA was detected in her urine, but none were detected in either PBLs or plasma. Four weeks after transplantation, $10^{1.9}$ copies of virus DNA were detected in 10^6 PBLs (Fig. 4B). Shortly after this, pp65 positive cells were detected in her blood. CMV DNA was not detected in her plasma during this follow up. Although the patient was asymptomatic, ganciclovir was administered prophylactically, starting when the antigenemia was first detected. In the six months because her transplantation, no CMV-related symptoms have occurred.

DISCUSSION

A real-time PCR assay was developed for quantifying the CMV genome load in clinical samples. Many methods for the virus titration have been developed and applied to clinical cases [Gleaves et al., 1985; Gerna et al., 1990; Veal et al., 1996; Boivin et al., 1997]. Most of these methods, however, are not always suitable for bedside use because of the time and labor required. The real-time PCR assay involves minimal handlings, is fast, has a wide dynamic range, and is accurate for clinical applications. Our results showed that the real-time PCR assay was as useful as the pp65 antigenemia assay, because they were highly correlated. Unlike the conventional qualitative PCR assay, that is not beneficial for terminating antiviral therapy [Gerna et al., 1991], this assay showed that the copy number of CMV DNA decreased and disappeared in response to anti-CMV therapy. Additionally, with this PCR method there is almost no room for bias due to subjective assessments by technicians. We suppose that this is an important difference from the antigenemia assay.

When the CMV load in the symptomatic and the asymptomatic patients was compared, there were significant differences in the rate of detecting CMV DNA in their PBLs in both immunocompromised and immunocompetent groups. Furthermore, the CMV DNA copy

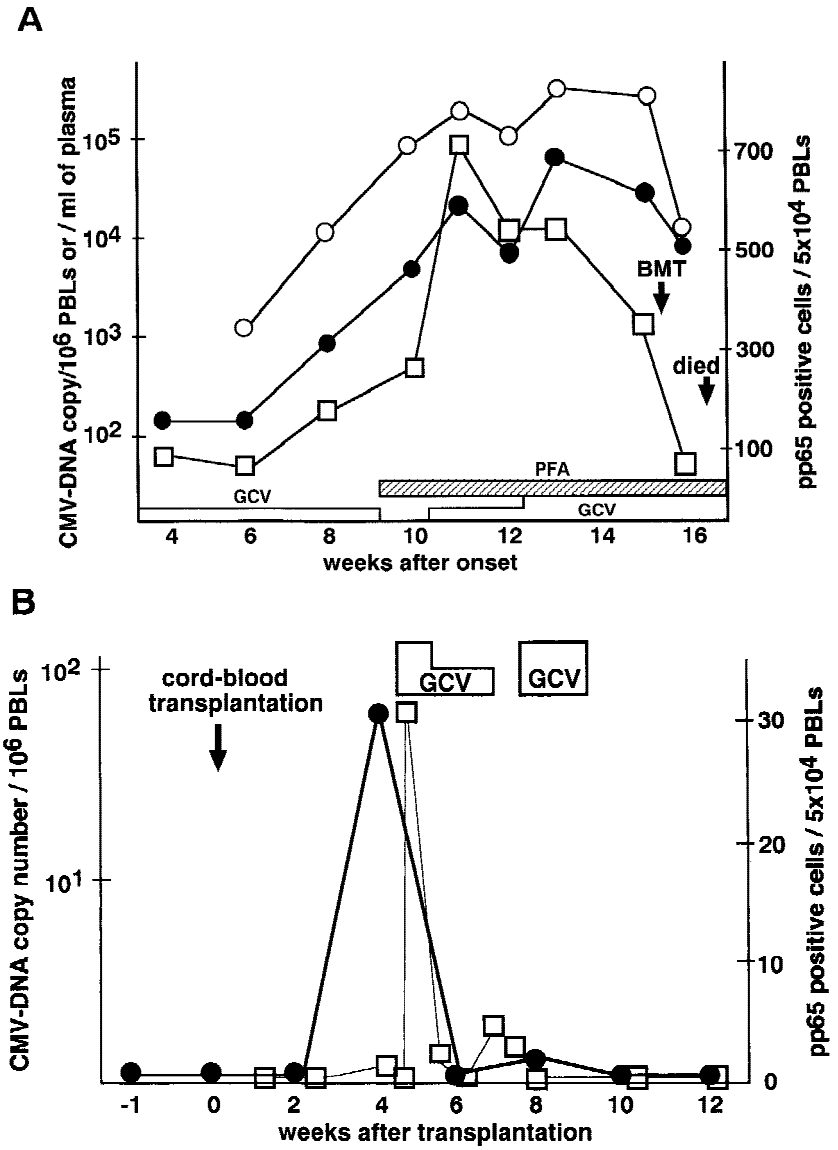

Fig. 4. Serial monitor of CMV load by the real-time PCR and antigenemia assay. Sequential samples were obtained from 2 clinical cases and analyzed to monitor the virus load. Closed circles; The CMV-DNA copy numbers in 10^6 PBLs by the real-time PCR assay. Open circles; The CMV-DNA copy numbers in 1 ml of plasma by the real-time PCR assay. Open squares; The pp65-antigen positive cell counts per 5×10^4 PBLs by the antigenemia assay. (A) A case of severe combined immunodeficiency infant with hepatitis and fulminant retinitis. (B) A case of acute myelogenic leukemia with cord-blood transplantation. CMV DNA was not detected in her plasma during this follow up. GCV, ganciclovir; PFA, foscarnet; BMT, bone marrow transplantation.

number of symptomatic patients was significantly larger than that of asymptomatic ones in the immunocompromised patients. As for plasma, most of samples from the immunocompromised-symptomatic group were positive for CMV DNA, whereas the virus genome was rarely detected in the asymptomatic group. There are many reports that detect virus DNA in the blood samples of patients with active CMV infections based on traditional qualitative PCR methods [Spector et al., 1992; Boeckh et al., 1997]. Such qualitative analyses, however, are not sufficiently useful for diagnosing CMV-related diseases in immunocompromised host because this virus sometimes reactivates asymptomatically in these patients. The real-time PCR assay will be very useful for quantifying CMV DNA in PBLs to distinguish patients with symptomatic infections that must be treated with antiviral agents from those with latent or inactive infections. Additionally, the detection and quantitation of CMV DNA in plasma would help this interpretation because the presence of viral DNA in plasma is highly specific for the symptomatic CMV infection as shown in this study.

In urine samples, there was a significant difference in the CMV DNA copy number between the symptomatic and asymptomatic groups. To our knowledge, there are only a few reports concerning the quantitation of CMV-DNA in urine [Cope et al., 1997]. Assessing the CMV DNA load in urine would help in the diagnosis of

symptomatic CMV infections. Our technique to quantify CMV DNA in urine could be applied to the prenatal diagnosis of symptomatic CMV infections, because amniotic fluid, that is usually used for the prenatal diagnosis, consists of fetus's urine. Recently, Lazzarotto et al. reported the prenatal diagnosis of congenital CMV infection by isolating the virus and using a PCR method [Lazzarotto et al., 1998]. For prenatal diagnosis using amniotic fluid, rapidity and reliability are important in making a decision on fetal treatment or termination of the pregnancy [Lazzarotto et al., 1998; Negishi. et al., 1998]. The real-time PCR assay is a suitable method for this purpose.

In Patient A, the decrease in the pp65-positive cells was greater than the drop in the CMV DNA copy number in PBLs and plasma. It is recognized that the pp65 antigen in neutrophils is the virus tegument protein that is captured by, not newly synthesized in [Grundy et al., 1998]. The function of his neutrophils may be damaged by the conditioning therapy for his bone marrow transplantation or the severe complication. On the other hand, in patient B, the peak of CMV DNA was earlier than that of the pp65 antigenemia. The pp65 positive cells increased after the number of neutrophils recovered in this patient. When neutrophil function is impaired or the number of neutrophils decreases, the antigenemia assay may not be sufficient to estimate active CMV replication. Limaye et al. showed that in blood stem cell transplantation recipients, CMV diseases occurred even before engraftment [Limaye et al., 1997]. Although further comparison of the quantitative PCR and antigenemia assays is required [Bossart et al., 1997; Gerna et al., 1998], it might be beneficial to estimate the CMV load using the real-time PCR assay in such special situations.

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